A Video Method to Study Drosophila Sleep

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Study Objectives: To use video to determine the accuracy of the infrared beam-splitting method for measuring sleep in Drosophila and to determine the effect of time of day, sex, genotype, and age on sleep measurements.

Design: A digital image analysis method based on frame subtraction principle was developed to distinguish a quiescent from a moving fly. Data obtained using this method were compared with data obtained using the Drosophila Activity Monitoring System (DAMS). The location of the fly was identified based on its centroid location in the subtracted images.

Measurements and Results: The error associated with the identification of total sleep using DAMS ranged from 7% to 95% and depended on genotype, sex, age, and time of day. The degree of the total sleep error was dependent on genotype during the daytime ($P < 0.001$) and was dependent on age during both the daytime and the nighttime ($P < 0.001$ for both). The DAMS method overestimated sleep bout duration during both the day and night, and the degree of these errors was genotype dependent ($P < 0.001$). Brief movements that occur during sleep bouts can be accurately identified using video. Both video and DAMS detected a homeostatic response to sleep deprivation.

Conclusions: Video digital analysis is more accurate than DAMS in fly sleep measurements. In particular, conclusions drawn from DAMS measurements regarding daytime sleep and sleep architecture should be made with caution. Video analysis also permits the assessment of fly position and brief movements during sleep.

Keywords: Video analysis, Drosophila, sleep, deprivation, age

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BEHAVIORAL QUIESCENCE IN DROSOPHILA SHARES FUNDAMENTAL SIMILARITIES TO MAMMALIAN SLEEP.[1,2] BOTH ARE CONTROLLED BY CIRCADIAN and homeostatic processes, and, during both mammalian sleep and Drosophila quiescence, the sensory arousal threshold is increased compared to during the active periods.[1-3] This similarity extends to the biochemical level. The wake-promoting drugs caffeine and modafinil decrease quiescence in Drosophila[1,2,4]; the sleep-promoting drug hydroxyzine increases quiescence in Drosophila,[2] and regulation of quiescence shares genetic regulation with regulation of mammalian sleep.[5-11] The study of the genetic basis of sleep in arthropods is therefore bound to shed light on our understanding of mammalian sleep regulation.

The standard method for determining Drosophila sleep uses an infrared beam; when the fly's body interrupts this beam's path, a signal is recorded.[12] A system now in wide use is called the Drosophila Activity Monitoring System or DAMS (Tri Kinetics, Waltham, MA) in which the infrared beam is directed through the center of a glass tube, which is approximately 6 cm long. Using DAMS, the fly's gross activity patterns can be monitored over several days. Although powerful for studying gross activity patterns, such as circadian periodicity, the DAMS system has certain limitations for studying sleep. First, it is insensitive to small fly movements. The error caused by small fly movements, which are not recorded as interruptions of sleep bouts because the fly does not cross the path of the infrared beam, would particularly impact measures of sleep architecture, such as sleep bout duration. This error could be influenced by the preferred position of the fly in the tube, since movements made by the fly when it is farther away from the beam will not result in a beam break and therefore not be detected. Second, the degree of fly movement is not addressed by the DAMS system. Small fly movements are only recorded if the fly is located near the center of the tube, and large movements along the length of the tube are recorded as only a single beam break. Third, the location of the fly is not recorded with the use of the DAMS system. This is relevant because periods of no beam breaks may correspond to time that the fly is eating at one end of the tube, or trying to escape at the other end, rather than sleeping. These are important experimental issues because a perturbation, eg, a genetic difference, might affect the DAMS-based measurements of sleep by changing the preferred location of the fly rather than by affecting quiescence.

To address these limitations, we have developed a digital video analysis method to identify behavioral quiescence. To identify behavioral quiescence with high spatial resolution, and without the confounding variable of a changing object shape, we developed a new method using subtraction analysis of video images for distinguishing a quiescent from a moving animal. We have determined that the error associated with the use of DAMS to identify sleep is influenced by sex, genotype, and age. The error of DAMS for identification of sleep is greater during the day when examining total sleep and is large for sleep bout duration determinations regardless of time of day. We use video to show that the fly's preferred position in the tube is influenced by genotype, a finding that might explain the effect of genotype on the accuracy of sleep determination using DAMS. Using video, we have identified distinct small movements that occur during sleep. Finally, we have confirmed, using video, the previously described homeostatic response to sleep deprivation.

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METHODS

Fly Strains

The Canton-S (CS) strain was a gift from Joan Hendricks[4] and was sib mated for 20 generations before use in this study to maximize homozygosity of this strain. The *white* strain w^{1118ex} was the background for a mutagenesis study[13] and was obtained from the Bloomington Stock Center (http://flystocks.bio.indiana.edu/). The wRR strain was a gift from William Joiner, who created it by crossing the *white* mutation into the RC 1 strain (Bloomington Stock Center) followed by repeated backcrossing to the RC 1 strain to maximize homozygosity.

Aged Flies

Female w^{1118ex} flies were kept in groups of 8 to 10 animals (initial population) on standard cornmeal agar media under 12-hour light:12-hour dark conditions at 25˚C and constant humidity for 38 days with transfer to new vials every 2 to 3 days. The aged flies were then transferred individually to monitor tubes, as described below, and maintained for 5 days before their behavior was recorded. By measuring fly survival over time, we determined that at 45 days, the age of the old flies when their behavior was recorded, 90 % of the w^{1118ex} fly population had died, indicating that the surviving flies used for the experiment are likely to be physiologically aged.

Fly Sleep Recording

Flies were collected upon eclosion and kept overnight on standard cornmeal agar media, then transferred individually under CO_2 anesthesia into tubes 6 cm long containing 5% sucrose/1% agar as a food source at one end of the tube and plugged with yarn at the other end to prevent escape and to allow ample diffusion of gases. Behavior recordings of fly sleep were done in Precision Instruments 818 incubators equipped with two 1.22-meter long fluorescent bulbs mounted vertically on the door under 12 hours white light:12 hours infrared light (wavelength 950 nm) at 25˚C and constant humidity for 5 days before behavior was recorded. Lux levels during the 12 hours of light ranged from 892 lux to 425 lux depending on the incubator and the position in the incubator. Monitors were positioned perpendicular to the light source on the incubator door to ensure that each monitor tube had equal illumination along its length. Infrared illumination was provided using a PRH-5218 light-emitting diode array (Merit Li-Lin, Arcadia, CA). ZT 0 is defined as the time of lights on, and ZT 12 as the time of lights off.

Sleep Deprivation

Seven-day-old CS females were monitored by video and DAMS continuously from ZT 0 of the day before deprivation to ZT 24 of the day after deprivation, i.e. for 48 hours, except during the actual deprivation period. During deprivation, flies were visually monitored to ensure they did not sleep in this period and continued to be recorded by DAMS. Flies were sleep deprived for 3 or 6 hours beginning at ZT 20 or ZT 17, respectively, and ending at ZT 23. A second control group of flies was treated identically but allowed to behave normally, i.e. were undisturbed, during the deprivation period. These served as controls. The deprivation was performed by gently shaking the flies in their individual monitor tubes in the DAMS monitor by hand under red safety lighting (Kodak).

Image Capture

For the direct comparison with the DAMS, the monitor tubes containing single flies were placed in the system per the manufacturer's recommendations. DAMS data were collected in 30-second bins. To maximize the contrast between the flies and the background, the area of the DAMS monitor under the tubes was painted white using Wite-Out (Bic USA, Milford, CT). To image the flies, a Retiga 1300i or 2000R camera (QImaging, Surrey, BC) was mounted 59 or 32 cm above the DAMS tube, respectively. Images were captured using Matlab (The Mathworks Inc., Natick, MA) for 24 hours, ie, a complete 12-hour light:12-hour infrared cycle. The rate of image capture was once every 5 seconds (0.2 frames per second). Camera exposure times for each frame were 50 milliseconds during lights on and 150 milliseconds under infrared illumination. To ensure constant illumination between captured video frames, the exposure times were multiples of 1/60 seconds, the time of a single cycle of the fluorescent light source.

Digital Video Analysis

Custom software written with a combination of Matlab and C+ computer languages was used to analyze the video images. The method of subtraction analysis of video images is illustrated in Figure 1. Corresponding pixels from 2 temporally adjacent images are subtracted and a resulting DIFFERENCE image is generated. Each pixel in the DIFFERENCE image has the value $GS(XiYj)=[(GS_2(XiYj)-GS_1(XiYj))/2]+127$, where $GS(XiYj)$ is the DIFFERENCE image grayscale value centered around a value of 127 at pixels X position i and Y position j and GS_2 and GS_1 are the grayscale values at that same pixel for the second and first video frames, respectively, in a pair of temporally adjacent frames. The images were digitized using 8 bits per pixel; therefore, the range in grayscale values for a single pixel was from 0 to 255. Areas of interest corresponding to each monitor tube, ie, each fly, were analyzed separately. Gray pixels, ones in which motion did not occur, have values close to 127. They are not exactly 127 because of noise in image acquisition. Since the degree of noise varied between incubators and cameras, for every experiment, the degree of this noise was determined by analyzing a portion of the image that was outside the monitoring tubes and therefore contained no movement. To illustrate the magnitude of noise, distributions of grayscale values from subtraction analysis of a tube that contained a dead fly and from a tube that contained a live fly are shown in Supplemental Figure 1 (supplemental data is available on the *Sleep* website at www.journalsleep.org). The range of grayscale values for the dead animal corresponds to a change in grayscale value close to ± 20 from 127. This was also true for areas outside of the monitoring tubes (data not shown). Darker pixels, ones that represent the location to which the fly had moved, have values less than 108, whereas whiter pixels, ones that represent the location from which the fly moved, have

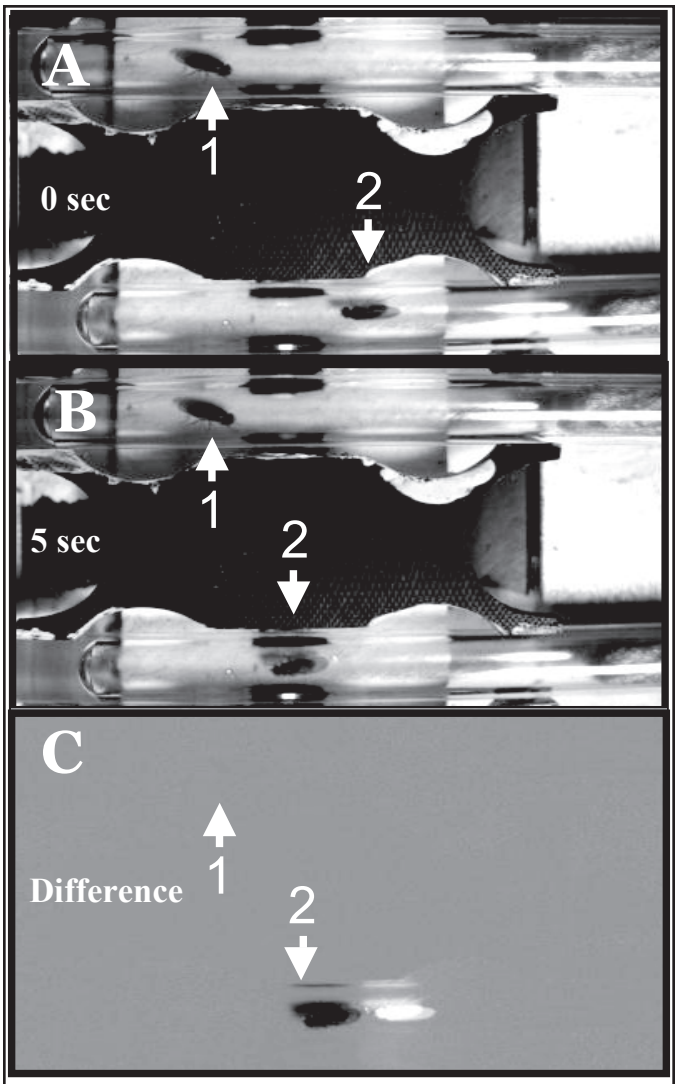

Figure 1—(A) Image of 2 flies in recording chambers. Positions of the flies are marked with white arrows. (B) Image of the same 2 flies captured 5 seconds after (A) fly #2 had moved and fly #1 had not. (C) The difference of (B) - (A) as grayscale values. Movement of fly #2 resulted in a white image (high grayscale values) at its previous location in the prior frame and a dark image (low grayscale values) at its new location in the current frame (marked by a white arrow). Fly #1, which had not moved, produced no white or dark pixels. The infrared beam of the Drosophila Activity Monitoring System monitor is visible in (A) and (B) as a bright spot in the lower portion of each figure in the middle of the tube. Fly #2 ends its movement into the electronic beam in (B).

values greater than 148. A single camera was used to analyze 8 flies housed in the DAMS monitors.

The number of white pixels and of dark pixels present in the subtracted image was determined. The larger of these 2 numbers was considered to be the number of pixels moved by the fly between the 2 images.

The location of the fly was determined by identifying the centroid of the dark object in the subtracted image, where the centroid is defined as the geometric center of the smallest rectangle that can enclose the dark object. If the fly did not move in a 5-second epoch, the position was assigned as the position in the last frame in which the fly had moved.

Analysis of Sleep Architecture

Quiescence measurements obtained using the DAMS and video systems were analyzed using custom software written using C and Ruby computer languages to obtain the following parameters: total sleep (where sleep is defined at 5 minutes or more of quiescence), sleep bout duration, number of pixels moved, and location of the fly in the monitor tube. The fly was considered to be asleep if it spent at least 5 minutes with no pixels moved. We have based the duration of quiescence upon prior literature in which fly sleep was defined as a minimum of 5 minutes of rest, based on changes in arousal threshold measured using 3 distinct methods: visual scoring of videotaped flies,[2] analysis of DAMS data,[3] and measurements of thoracic muscle activity of immobilized flies.[14]

Visual Validation of Video Analysis

Two young flies of each sex and genotype and 2 aged female flies of the w^{1118} genotype were chosen at random for direct visual validation of the automated video analysis. For each of these flies, the 30-minute video interval in which there was the largest differences between the DAMS and video-based sleep determinations in a 12-hour light period were visually inspected. This process was repeated for the 12-hour dark period. The videos were observed by manually advancing individual frames. A trained observer who was blinded to the results of the DAMS and video scoring determined whether or not the fly moved between each pair of images. Using this analysis, we determined that the automated video analysis missed visually detectable small movements 7.4% of the time (the range was 5.0%-10.6%) in the flies we sampled. Movements not detected by the automated video analysis consisted of wing flicks or of isolated leg movement or proboscis extensions, which did not generate grayscale values outside of the noise range in the difference image.

Statistical Methods

Mixed-Model Analysis of Variance

Mixed-model analysis of variance (ANOVA) was used to compare sleep parameters estimated using video analysis and DAMS. This method optimally accounts for correlations between sleep parameters obtained from the same fly by including the fly as a random effect when estimating fixed effects. The primary fixed effect was method of measurement (DAMS vs video). Specific ANOVA models also included fixed effects for genotype, sex, and age. Interactions between these factors and method of measurement were included to assess whether differences between DAMS and video depended on genotype, sex, or age. Least-squares estimates of contrasts reflecting adjusted differences were estimated from the ANOVA models along with appropriate standard errors. Statistical significance levels for pair-wise contrasts were adjusted for multiple comparisons using a statistically powerful simulation method that is robust and accounts for the correlation structure within the set of hypotheses tested.[15,16] To account for the low power of tests for interactions, interactions were considered significant if the

multiplicity-adjusted P value was less than 0.10. Simple effects were assessed when interactions were significant (ie, contrasts for one factor were made within levels of another factor). Multiplicity-adjusted P values for main effects and simple effects were considered statistically significant if their multiplicity-adjusted P values were less than 0.05. Similar methods were used to assess the effects of age and sleep deprivation. Mixed-model ANOVA and multiplicity adjustments were implemented using the SAS procedure Proc Mixed.[17]

Group Comparisons of Maintained Wakefulness

The distributions of the durations of maintained wakefulness during the first wake bout at the beginning of the day were compared among experimental groups. The first wake bout was defined as the first wake bout initiated after lights on, if the fly was asleep at ZT 0, or the first wake bout that had begun within 1 hour before lights on if the fly was already awake at ZT 0. A wake bout was considered terminated when the fly spent 5 or more minutes quiescent. The duration of maintained wakefulness, which is also the latency to the first sleep bout following sleep deprivation, was interpreted as a measure of how sleepy the fly was (ie, propensity to initiate sleep) following deprivation. The duration of sleep latency was compared with that in control animals that were not sleep deprived. Kaplan-Meier survival curves were constructed to graphically compare distributions of duration of sleep latency among groups of flies that were sleep deprived for 3 or 6 hours relative to controls that were not sleep deprived. A log-rank statistic was used to assess the statistical significance of group differences among the distributions of this duration.

RESULTS

Video Analysis Resolves Spatial Discontinuity of DAMS

The chief limitation of DAMS is that it is spatially discontinuous: only when the fly crosses the middle of the tube will a movement be detected. To monitor movement in a spatially continuous fashion and therefore more accurately distinguish quiescent from moving flies, we developed a method based on subtraction of pairs of digital images. This method is distinct from those available in most commercial motion-tracking systems, which track the location of the centroid of an object. Centroid tracking is based on the assumption that the tracked object has a constant shape and size. This assumption is invalid for the fruit fly, which has a highly variable shape and size depending its orientation and location in the tube. Therefore, centroid tracking, although adequate for detecting macroscopic movement and for the identification of location, does not accurately identify small movements of the animal. A variation of this frame subtraction method was previously used to identify quiescence in *C. elegans*.[18] The principle is illustrated in Figure 1. Pairs of temporally adjacent images are subtracted. If there is no movement between the 2 frames, then the difference image will be all gray, and the grayscale value of the individual pixels will be close in value to 127. If the fly moves between the images, then dark pixels are detected at the fly's new location and white pixels at its old location (Figure 1C). The spatial resolution for detecting movement with this method approaches 1 pixel, which, in our recording conditions, corresponds to 189 microns, approximately 9% of the body length of a fly. Rarely, single-pixel movements that do not result in sufficiently different grayscale values are missed by the automated subtraction video analysis but can be detected by visual inspection of the frames (see Methods).

In addition to determining whether or not the fly had moved, our method allows an estimation of the magnitude of movement based on the number of pixels moved between pairs of images. Finally, the location of the fly in the tube can be determined by identifying the centroid of the dark object (the new location of the fly) in the difference image when the fly has moved.

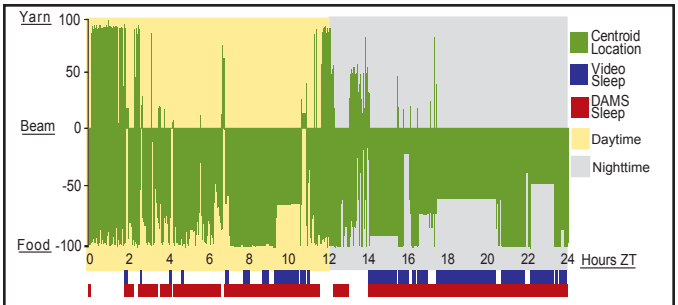

Figure 2—The Drosophila Activity Monitoring System (DAMS) misses movements away from the beam. The location of a single w^{1118} female monitored simultaneously by DAMS and video for 24 hours is shown. The infrared beam of the DAMS monitor is the reference point and equals 0 on the Y axis. The distance toward the yarn or toward the food in pixels is shown as a green bar for each 5-second video capture. The X axis is the time of day in 2-hour intervals from lights on (ZT 0). Daytime (ZT 0 to T 12) is indicated by the yellow background. Nighttime (ZT 12 to ZT 24) is indicated by the light gray background. The periods identified as sleep using video are shown as blue bars and the periods identified as sleep using DAMS are shown as red bars below the X axis. There are frequent instances, particularly during the day, in which the DAMS method considers the fly to be asleep while video does not. These periods usually correspond to times when the fly is moving in the space between the food and the beam.

DAMS Misses Movements Away From the Infrared Beam

Visual inspection of the videos shows that, as expected, there are frequent instances in which the fly moves but does not cross the middle of the tube and, therefore, is erroneously considered quiescent by the DAMS method (see example, "Video recording of DAM 'sleep' bout" at www.sleepgene.org/). Indeed, based on the established criterion of 5 minutes or more of quiescence for identifying a sleeping fly,[2,3,19] there are numerous instances, particularly during the 12-hour light period (daytime), in which DAMS considers the fly to be sleeping whereas video analysis shows that the fly is in fact moving (Figure 2). These movements can involve distances of several body lengths (see Figure 2 and video). This disagreement between the 2 methods ranges in frequency from 8.6% ± 2.9% to 15.6% ± 5.7% of all epochs (Table 1) in a 24-hour period.

In addition to the discrepancy between DAMS and video caused by the misclassification of movement away from the

beam as sleep by DAMS, we were surprised to also encounter a rare discrepancy in the opposite direction, ie, identification of movement by DAMS, in which the video detected no movement. This type of discrepancy, which happened less than 2% of the time, corresponded to times when the fly was immobile while sitting in or near the path of the infrared beam (see location of fly for different types of errors in Table 1). We therefore suspect that brief and tiny movements of the fly's wings, legs, or antennae broke the path of the beam, but these movements were either smaller than 189 microns—the spatial resolution of the video—and hence did not result in a sufficiently large grayscale difference to be identified as movements, or were movements briefer than 5 seconds, the temporal resolution of the video.

DAMS Overestimates Total Sleep

The missed movements by DAMS lead to an overestimation of total sleep in a 24-hour period (Table 2). Importantly, the genotype of the fly affects the degree of this error, as indicated by a significant GENOTYPE x METHOD interaction ($p \leq 0.001$) in a mixed-model ANOVA (Table 3). As one might expect from missing movements, DAMS also overestimates mean sleep bout duration for a 24-hour period ($P < 0.001$) (Tables 2 and 3).

The Greatest DAMS Error in Total Sleep Occurs During the Daytime

Prior studies have shown an effect of sex on sleep parameters, particularly during the daytime period.[19-21] Moreover, some experimental perturbations have been described to affect daytime but not nighttime sleep.[19,22] Therefore, it is important to know the relative contribution of daytime sleep and nighttime sleep measurements to the DAMS' overall error for the determination of sleep parameters. As suggested by the single-fly example shown in Figure 2, the daytime total sleep measurement by DAMS was found to be a much greater component of the error in total sleep determination over a 24-hour period: DAMS-based measurements identified 39% to 95% more sleep than did video measurements (Tables 2 and 3). The DAMS error in nighttime total sleep measurements (Figure 3B) is smaller, at 7% to 21%, than is the error in daytime measurements yet is still significant ($P < 0.001$) (Table 3). There was an effect of GENOTYPE for both daytime ($P < 0.001$) and nighttime total sleep ($P < 0.001$), indicating that there are clear sleep differences between these 3 wild-type strains. For daytime total sleep determination, the genotype of the fly affected the degree of error (Figure 3A), as indicated by a significant METHOD x GENOTYPE interaction using a mixed-model ANOVA ($P < 0.001$) (Table 3). Consistent with findings from prior studies, these results showed that sex affected daytime total sleep ($P < 0.001$), and the degree of this sex effect was dependent on method, as indicated by a METHOD x SEX interaction ($P < 0.001$) (Table 3).

Measurements of Sleep Bout Duration Is More Precise Using Video

The ability of video to accurately identify the time of sleep onset and offset leads to improved accuracy in the determination

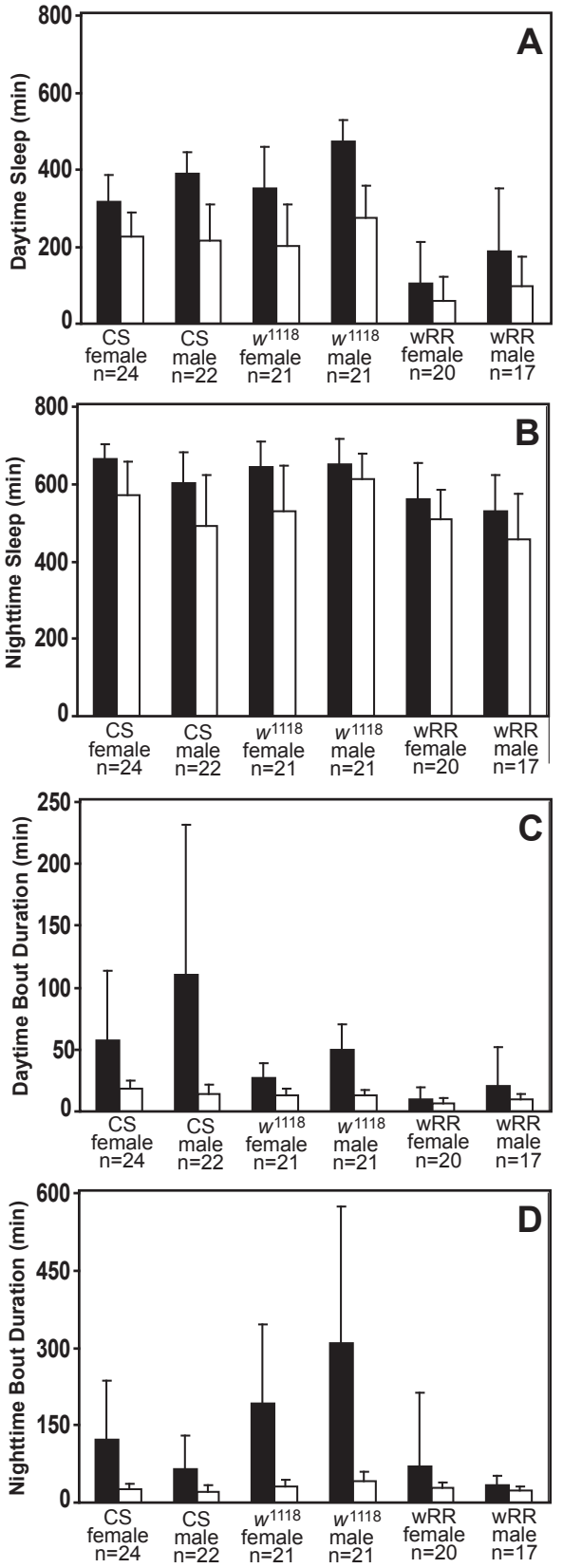

Figure 3—The Drosophila Activity Monitoring System (DAMS) overestimates sleep parameters for Canton-S (CS), w^{1118}, and wRR flies of different sexes. Shown is the mean ± SD for (A) Daytime total sleep. (B) Nighttime total sleep (C) Daytime mean sleep bout duration (D) Nighttime mean sleep bout duration. Black bars = measurements by DAMS, white bar = measurements by video.

Table 1—Location of the Fly During Discrepancies and Occurrence Rate of Discrepancies Between Video and DAM Analysis

			DAMS = Sleep Video = Wake		DAMS = Wake Video = Sleep	
Genotype	**Sex**	**No.**	**Pixel distance from beam**	**Occurrence, %**	**Pixel distance from beam**	**Occurrence, %**
Canton-S	female	9	38.3 ± 6.2	8.6 ± 2.9	11.3 ± 7.8	0.53 ± 0.47
Canton-S	male	8	51.8 ± 7.3	10.1 ± 3.6	11.0 ± 1.8	0.81 ± 0.59
w^{1118}	female	8	54.7 ± 11.5	14.6 ± 10.4	8.7 ± 3.6	0.77 ± 1.8
w^{1118}	male	7	63.2 ± 19.9	15.6 ± 5.7	6.8 ± 1.1	0.29 ± 0.47
*w*RR	female	9	44.0 ± 11.7	9.6 ± 10.7	6.6 ± 2.3	1.81 ± 1.26
wRR	male	7	28.9 ± 5.6	9.2 ± 5.8	3.1 ± 2.6	0.71 ± 0.68

Data are presented as mean ± SD. The flies' distance from the beam determines the type of error. The 2 types of discrepancies between Drosophila Activity Monitoring System (DAMS) and video in sleep determination are shown for both sexes and for 2 genotypes. Pixel distance from beam is the distance of the centroid of the fly from the beam in pixels when there was a discrepancy between DAMS and video. The occurrence, %, refers to the percentage of all epochs in which the type of error occurs in a 24-hour period. A single fly is 13 to 14 pixels in length.

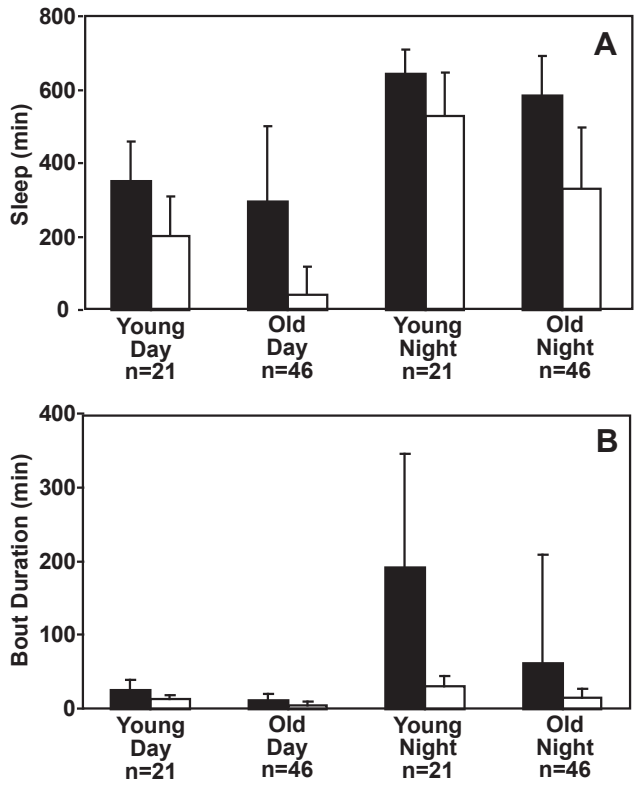

Figure 4—The Drosophila Activity Monitoring System (DAMS) overestimates both daytime and nighttime total sleep in old animals. Average ± SD Total sleep (A) and sleep bout duration (B) as determine by DAMS (black) and video (white) are shown for young (7 days) and old (45 days) w^{1118} females.

of sleep bout durations (Figure 3, Table 2). In addition to being more accurate, sleep bout durations obtained using video are less variable than those obtained using DAMS (Table 2). The DAMS-based measurements of the mean sleep bout duration were 63% to 702% greater during the day and 48% to 652% greater during the night than the simultaneous video-based measurements (Table 2). In addition, there was a METHOD x GENOTYPE interaction effect on the error associated with DAMS-based measurements of sleep bout duration (Table 3). This indicates that assessment of genotype effects on daytime and nighttime sleep bout durations using DAMS should be interpreted with caution.

The Genotype Effect May Be a Function of Preferred Location in the Monitor

We hypothesized that the GENOTYPE x METHOD interactions for daytime total sleep and nighttime mean sleep bout duration detected in the mixed-model ANOVAs (Table 3) can be explained by a genotype-dependent difference in use of the space available to the fly in the recording chamber. To test for this possibility, we determined the distributions of locations in the monitor tube for each of the 3 genotypes, during day and during night, during wake and sleep behavior, and for both males and females (Supplemental Figure 2). Inspection of this distribution leads to 2 conclusions. First, the flies distribute differently in the tube depending on their behavioral state. When awake, the flies distribute approximately equally across the tube, with a slight preference for the ends of the tubes. This preference may reflect the fact that, when awake, the fly will prefer to engage in either eating behavior at one end of the tube or escape behavior at the other end. In contrast with the distribution during wake behavior, during sleep behavior, flies of the CS and w^{1118} genotypes prefer a location closer to the food than to the yarn. This is true for both males and females. Being close to the food when asleep may allow for minimal movement and, therefore, minimal caloric expenditure requirement for a snack during a sleep bout.

The second conclusion is that there are differences in the distributions between genotypes, particularly during sleep. Whereas CS and w^{1118} flies have a propensity to sleep closer to the food than to the yarn end of the tube, wRR flies do not show this preference. wRR males have a preferred sleep position in the middle of the tube, close to the infrared beam. This distribution of wRR flies may explain why sleep bout duration measurements obtained in this strain using DAMS are more accurate than in the other genotypes (Table 2)

Age Increases the Error Associated With DAMS Measurements

Although Drosophila has been used as a model system for the study of aging, only recently has aging been shown to affect

Table 2—Comparison of Sleep Parameters as Determined by Video and DAMS Analysis

Genotype	Method	Total sleep, min	CV	Difference, %	Mean sleep bout, min	CV	Difference, %
24-Hour							
CS female	Video	799.2 ± 127.4	15.9	22.6	21.7 ± 6.8	31.3	221.5
	DAMS	979.6 ± 99.4	10.1		69.8 ± 58.5	83.8	
CS male	Video	708.2 ± 200.9	28.4	40.5	17.2 ± 6.6	38.4	248.4
	DAMS	995.1 ± 115.6	11.6		59.9 ± 47.6	79.5	
w^{1118} female	Video	733.3 ± 185.2	25.2	35.6	22.1 ± 6.4	28.9	153.1
	DAMS	994.7 ± 142.3	14.3		56.1 ± 27.3	48.7	
w^{1118} male	Video	887.3 ± 124.5	14.0	26.7	23.5 ± 6.9	29.4	259.9
	DAMS	1124.1 ± 83.9	7.5		84.6 ± 45.0	53.2	
wRR female	Video	567.5 ± 98.5	17.3	16.9	22.8 ± 7.6	33.3	37.1
	DAMS	663.4 ± 161.9	24.4		31.2 ± 18.1	58.0	
wRR male	Video	554.3 ± 147.8	26.7	29.9	19.0 ± 5.2	27.4	55.9
	DAMS	719.8 ± 204.5	28.4		29.6 ± 21.9	74.0	
Daytime							
CS female	Video	227.7 ± 60.9	26.7	38.7	18.4 ± 6.4	34.8	210.6
	DAMS	316.0 ± 70.5	22.3		57.3 ± 56.9	99.3	
CS male	Video	217.0 ± 92.3	42.5	80.3	13.8 ± 7.8	56.5	702.0
	DAMS	391.2 ± 53.7	13.7		110.4 ± 121.0	109.0	
w^{1118} female	Video	203.1 ± 105.1	51.7	72.9	13.3 ± 5.1	38.3	100.8
	DAMS	351.2 ± 107.9	30.7		26.8 ± 12.5	46.6	
w^{1118} male	Video	275.9 ± 83.5	30.3	71.2	13.0 ± 4.7	36.1	278.5
	DAMS	472.5 ± 55.3	11.7		49.3 ± 21.3	43.2	
wRR female	Video	59.9 ± 61.2	102.0	73.3	6.2 ± 4.6	74.2	62.7
	DAMS	103.9 ± 109.1	105.0		10.1 ± 9.4	93.1	
wRR male	Video	97.0 ± 77.3	79.7	94.8	9.4 ± 5.1	54.3	123.9
	DAMS	189.0 ± 161.0	85.2		21.1 ± 30.9	146.0	
Nighttime							
CS female	Video	571.4 ± 87.1	15.2	16.1	25.5 ± 11.3	45.5	374.1
	DAMS	663.6 ± 41.1	6.2		120.9 ± 114.5	94.7	
CS male	Video	491.2 ± 133.3	27.1	22.9	20.8 ± 13.0	62.5	209.9
	DAMS	603.9 ± 79.0	13.1		64.6 ± 65.6	101.0	
w^{1118} female	Video	530.2 ± 117.7	22.2	21.4	31.0 ± 13.9	44.8	519.1
	DAMS	643.5 ± 67.2	10.4		191.9 ± 154.3	80.5	
w^{1118} male	Video	611.4 ± 66.9	10.9	6.6	41.1 ± 18.5	45.0	652.1
	DAMS	651.6 ± 66.3	10.2		309.5 ± 264.0	85.3	
wRR female	Video	507.6 ± 78.8	15.5	10.2	27.4 ± 10.5	38.3	159.0
	DAMS	559.5 ± 95.4	17.1		71.0 ± 142.8	201.0	
wRR male	Video	457.3 ± 116.1	25.4	16.1	23.4 ± 8.9	38.0	47.9
	DAMS	530.9 ± 91.1	17.1		34.7 ± 17.8	51.3	

The Drosophila Activity Monitoring System (DAMS) consistently overestimates sleep parameters. Video and DAMS measurements of total sleep and the mean sleep bout duration for 24 hours, daytime, and nighttime are shown for 6 sex and genotype combinations of flies monitored by video and DAMS. Difference, %, refers to the percentage increase in DAMS mean values compared with the video mean value. CV is the coefficient of variation.

Drosophila sleep.[23] Comparing fly sleep measurements at different ages therefore requires an understanding of the effect of method on these measurements. We assessed the effect of aging upon the degree of error associated with DAMS estimations of sleep parameters using a 5-minute definition of sleep. DAMS significantly overestimates both daytime and nighttime total sleep in 45-day-old females ($P = 0.001$ and $P < 0.001$, respectively) (Figure 4A). A mixed-model ANOVA detected a significant AGE x METHOD interaction for both daytime and nighttime total sleep ($P = 0.008$ and $P < 0.001$, respectively) and sleep bout duration ($P = 0.004$ and $P = 0.004$, respectively), indicating that the degree of error between DAMS and video was dependent upon the age of the animal (Figure 4B). In particular, there is a much larger difference (76.8%) in the estimate of nighttime sleep in old flies compared with young flies (21.4%).

A Homeostatic Response to Sleep Deprivation Is Confirmed Using Video

The homeostatic property of sleep refers to the fact that, following a period of enforced wakefulness, animals respond with elevated sleep pressure, as reflected by shortened sleep latency, more consolidated sleep, and increased sleep at a time of day when flies are normally mostly awake. Several prior studies have used DAMS to demonstrate a fly's homeostatic response to sleep deprivation. Given the above results, it remained possible that this effect was a consequence of an altered distribution of the flies in the monitor tubes rather than altered sleep. We therefore used video to assess for a homeostatic response to sleep deprivation and to directly compare the 2 methods in this assessment. The CS females were sleep deprived for 3 or

Table 3— Results of Mixed-Model ANOVA for Video Versus DAMS Determinations

Effect	24-hour Total Sleep	Mean Bout Duration 24 h	Daytime Total SleepTST	Daytime Mean Bout Duration	Nighttime Total Sleep	Nighttime Mean Bout Duration
Method	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Sex	0.058	0.661	**< 0.001**	**0.008**	0.128	0.744
Genotype	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Method X Sex	0.052	0.244	**< 0.001**	**0.005**	0.458	0.763
Method X Genotype	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.060	**< 0.001**
Sex X Genotype	**0.003**	**0.040**	0.125	0.371	**0.003**	**0.007**
Method X Sex X Genotype	0.094	0.118	0.550	0.118	**0.007**	**0.023**

Results of mixed-model analyses of variance (ANOVAs) for video versus Drosophila Activity Monitoring System (DAMS) determinations. P values for main effects and for all interactions are shown. Significant P values are in bold. Data are shown for 6 main variables. Separate mixed-model ANOVAs were estimated for each of the 6 sleep parameters, as listed on the title row of this table The table summarizes the statistical significance of the following effects on the sleep parameters: METHOD = DAMS compared with video, SEX = male compared with female, GENOTYPE = comparison of wRR, Canton-S (CS) and w^{1118}. The lower 4 rows show the results for all possible interactions of these 3 effects.

6 hours during their normal sleep period, ending at ZT 23. The flies were monitored using both DAMS and video during the baseline day and the recovery day. A mixed-model ANOVA was used to determine the contribution of METHOD (DAMS vs video), HOUR, and GROUP (control for 3 and 6 hours and sleep deprived for 3 and 6 hours) to the variance of total sleep. Consistent with prior studies showing a homeostatic response to sleep deprivation,[1-3] there was an effect of GROUP, ie, difference between control and sleep-deprived flies, (P = 0.040). Consistent with our results showing differences between DAMS and video-based measurements of sleep, there was an effect of METHOD (P < 0.001). There were, however, neither METHOD x GROUP nor METHOD x HOUR interactions (P = 0.106 and P = 0.988, respectively), indicating that assessment of the effect of sleep deprivation is not significantly affected by the method. Specifically, both video and DAMS detect an increase in sleep during the 2-hour period beginning at ZT 2 following 3 hours of deprivation and an increase in sleep for the 2-hour period beginning at ZT 1 following 6 hours of deprivation.

We also examined the effect of sleep deprivation on sleep latency beginning at ZT 0 (see Methods). Figure 5A contains Kaplan-Meier survival curves that provide a graphic comparison among experimental groups in terms of their distributions of sleep latency durations as determined by video at diurnal times corresponding to the end of the sleep-deprivation intervention. The group differences among survival curves for control non–sleep-deprived groups and flies sleep deprived for 3 and 6 hours were different (log-rank $\chi^2 = 22.6$, df = 3, P < 0.001). The median durations of sleep latency were 74 minutes for flies sleep deprived 6 hours, 112 minutes for flies deprived 3 hours, and 227 minutes and 236 minutes for the 3 and 6 hour controls respectively. For comparison purposes, median durations of sleep latency on the day preceding the experimental day for these groups were not significantly different at 175 minutes, 215 minutes, 173 minutes, and 228 minutes, respectively (P = 0.09). Differences between controls during the matched postdeprivation periods were not different (P = 0.32). The hazard ratios, reflecting the instantaneous relative risk for sleep in the next moment relative to the group serving as the 3-hour deprivation control were 16.8 (95% confidence interval [CI] 4.2-67.4) and 4.1 (95% CI 1.2-13.8) for the 6- and 3-hour sleep-deprived groups, respectively. The difference between the 6-hour and 3-hour deprivation groups was also significant (P = 0.02, hazard ratio = 4.1, 95% CI 1.3-13.3). Thus, based on video, sleep deprivation produces large increases in the propensity to initiate sleep in a dose-dependent fashion.

Using DAMS to identify sleep, group differences were also significant (log-rank $\chi^2 = 15.4$, df = 3, P = 0.002), but the time to first sleep was much shorter for all groups, even the control groups. The median durations of maintained wakefulness were 12 minutes, 68 minutes, 113 minutes, and 140 minutes for flies sleep-deprived for 6 hours, for flies sleep deprived for 3 hours, and for the 2 control groups, respectively. Using DAMS, the hazard ratios of the deprived group relative to the 3-hour deprivation control group were 9.6 (95% CI 2.6-35.2) and 3.8 (95% CI 1.1-13.0) for the 6- and 3-hour sleep-deprived groups. The group difference between the 6-hour and 3-hour deprivation groups using DAMS was smaller than when using video and did not reach statistical significance (P = 0.09, hazard ratio = 2.5, 95% CI 0.9-7.3) (Figure 5B). At the current sample size (n = 7-8), there was a clear difference in sleep latency between 3 and 6 hours of sleep deprivation using video analysis but not when using DAMS. Thus, the misclassification of wake bouts using DAMS resulted in shorter estimates of the time to the first sleep bout and produced attenuated estimates of the dose responsiveness of the effects of sleep deprivation.

Video Recordings Identify Brief Movements During Sleep

Human sleep can be interrupted by brief arousals. These arousals are typically much shorter than the typical sleep bout and often correspond to brief movements in bed without overt behavior that suggests awareness. Such arousals have clinical significance, since they may underlie the pathophysiologic mechanism that results in sleepiness from intrinsic sleep disorders, such as obstructive sleep apnea and periodic limb movements of sleep.[24] We hypothesized that fruit flies also have brief movements during sleep that may not represent full transitions

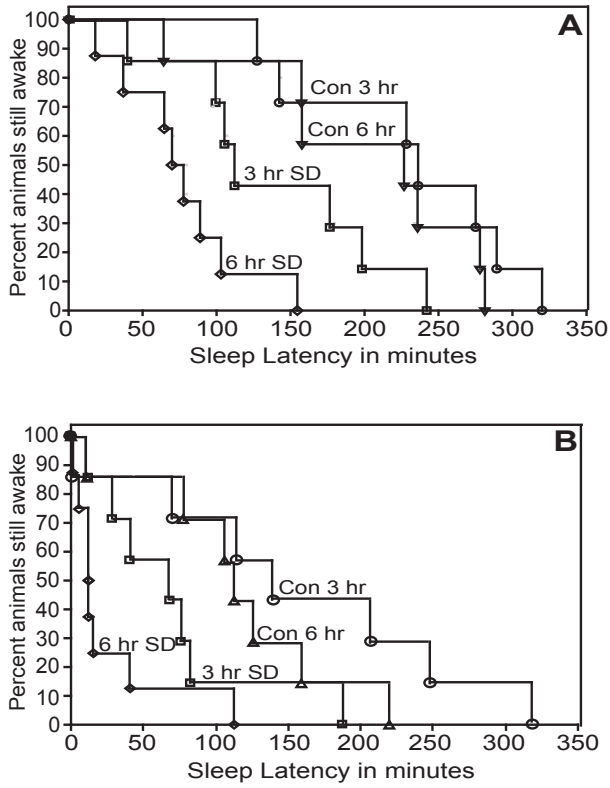

Figure 5—Distribution of duration of sleep latency following lights on are shown for 4 groups, 2 control groups (CON) and 2 sleep-deprivation (SD) groups. Data for Canton-S females are shown as Kaplan-Meier survival curves for video (A) and Drosophila Activity Monitoring System (DAMS) (B). The Y axis is the percentage of flies still awake after lights on. The X axis is the time in minutes from ZT 0. Con 3 hr [circle] = the control group for the 3-hour deprivation experiment (n=7). Con 6 hr [triangle] = the control group for the 6-hour experiment (n = 7). 3 hr SD [square] = group deprived from ZT 20 to ZT 23 (n=7). 6 hr SD [diamond] = group deprived from ZT 17 to ZT 23 (n=8).

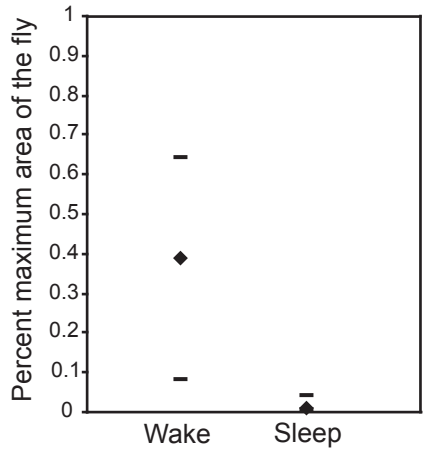

Figure 6—Movements within sleep bouts are considerably smaller than movements during wake bouts. Pixels moved per five second video acquisition (epoch) were normalized to the maximum pixel area of each w^{1118} female fly (n=8). The 25%, 50%, and 75% quartiles for all single 5-second movements as a percentage of total area of the fly are shown. Wake = acquisitions on either side are also movement. Sleep = the preceding and following epochs are at least 2.5 minutes of quiescence.

to the wake behavior state. If this were true, we would expect brief movements during sleep to be of small magnitude. We therefore inspected the distribution of pixels moved when these movements occurred in isolation during a single 5-second epoch and were flanked by at least 2.5 minutes of quiescence, ie, before and after the brief movement. That is, there was a total of 5 minutes or more of quiescence interrupted by only a single brief movement. Inspection of the data (Figure 6) shows that these movements are small, with a median of 1 pixel or 0.7% of total area of the fly in pixels moved per 5-second epoch. In contrast, when movements occurred in the context of wake behavior, the movements were larger and more variable (Figure 6), with a median of 70.5 pixels or 48.4% of total area of the fly in pixels moved in a 5-second epoch. Therefore, there are 2 movement types that depend on the context of the behavior in which the movement occurred: during sleep, the movements are small, whereas, during wake, these movements are large. This suggests that brief movements during sleep are a distinct behavior state from wake and might be brief arousals.

Because these brief movements can interrupt sleep bouts and, hence, might result in a lower estimation of total sleep and of sleep bout duration, we reanalyzed the data by allowing brief movements to occur during sleep bouts. Inclusion of brief movements as part of a sleep bout increases daytime total sleep by 2.7% to 10.5% and nighttime total sleep by 1.2% to 5.0% (Table 4). The sleep bout duration measurement is more significantly affected by allowing brief movements during sleep and is increased by 8.1% to 50.4%. This indicates that bout duration measurements are sensitive to brief small movements (Table 4). However, even when brief movements are included as part of the sleep bouts, ie, are considered not to disrupt a sleep bout, differences between video and DAMS-based sleep measurements persist, as indicated by a main effect of METHOD in a mixed-model ANOVA ($P < 0.001$) (Supplemental Table). The effect of GENOTYPE alone on sleep measurements is now seen only during daytime total sleep and daytime mean sleep bout duration ($P < 0.001$ and 0.005, respectively), suggesting that daytime sleep measurements are the ones most sensitive to the genetic background. There remains a strong METHOD x GENOTYPE interaction effect for daytime total sleep and for daytime and nighttime sleep bout duration, $P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively (Supplemental Table).

In summary, although inclusion of brief movements into sleep bouts does increase both total sleep and sleep bout duration, the differences observed between video and DAMS measurements of sleep hold.

DISCUSSION

We have developed a video method for studying sleep in Drosophila and have used this method to assess the accuracy and precision of the DAMS, the current widely used analysis tool for studying Drosophila sleep. DAMS-based measurements overestimate sleep amounts, in particular during the daytime. The determination of sleep architecture, eg, sleep bout duration, is particularly problematic. We note that certain experimental conditions, eg, sex, age, prior sleep deprivation, and genotypes,

Table 4—Total Sleep and Mean Sleep Bout Duration as Determined With (INCLUDED) and Without (EXCLUDED) Allowing Brief Movements to Interrupt a Sleep Bout

Genotype sex Age	N	Brief Movements During Sleep	DAYTIME Total Sleep	%	Mean Sleep Bout	%	NIGHTTIME Total Sleep	%	Mean Sleep Bout	%
CS Female	24	EXCLUDED	227.7 ± 60.9	2.7	18.4 ± 6.4	16.3	571.4 ± 87.1	2.1	25.5 ± 11.3	15.7
7 days		INCLUDED	233.8 ± 61.3		21.4 ± 8.8		583.4 ± 79.6		29.5 ± 12.6	
CS Male	22	EXCLUDED	217.0 ± 92.3	5.9	13.8 ± 7.8	18.1	491.2 ± 133.3	2.6	20.8 ± 13.0	17.8
7 days		INCLUDED	229.7 ± 84.7		16.3 ± 9.1		504.0 ± 125.5		24.5 ± 12.8	
w^{1118} female	21	EXCLUDED	203.1 ± 105.2	5.4	13.3 ± 5.1	15.8	530.2 ± 117.7	2.9	31.0 ± 14.0	32.9
7 days		INCLUDED	214.1 ± 105.0		15.4 ± 5.9		545.4 ± 107.0		41.2 ± 20.4	
w^{1118} male	21	EXCLUDED	275.9 ± 83.5	6.8	13.0 ± 4.7	22.3	611.4 ± 66.9	1.2	41.2 ± 18.5	43.9
7 days		INCLUDED	294.6 ± 81.5		15.9 ± 7.3		619.0 ± 63.3		59.3 ± 30.3	
wRR female	20	EXCLUDED	60.0 ± 61.2	10.5	6.2 ± 4.6	8.1	507.6 ± 78.8	1.4	27.4± 10.5	50.4
7days		INCLUDED	66.3 ± 68.8		6.7 ± 5.0		515.0 ± 77.3		41.2 ± 20.4	
wRR male	17	EXCLUDED	97.0 ± 77.3	9.5	9.4 ± 5.1	12.8	457.3 ± 116.1	2.3	23.4 ± 8.9	19.2
7 days		INCLUDED	106.2 ± 84.2		10.6 ± 5.3		467.9 ± 114.4		27.9 ± 12.4	
w^{1118} female	46	EXCLUDED	42.9 ± 75.3	10.0	5.2± 4.3	13.5	331.2 ± 165.2	5.0	16.1 ± 10.8	18.6
45 days		INCLUDED	47.2 ± 83.0		5.9 ± 5.2		347.9 ± 167.1		19.1 ± 15.6	

Mean ± SD are shown for daytime (ZT 0 to ZT 12) and nighttime (ZT 12 to ZT 24) parameters. No. refers to the number of animals used to determine values; %, the percentage increase in the average values when brief movements are included as sleep.

with large effects on sleep have been and can continue to be detected using DAMS. However, there are concerns regarding the ability of DAMS to detect smaller effects and to accurately characterize sleep architecture.

We found that, for some experimental variables, eg, effect of genotype on total sleep, effect of sex on sleep architecture, and effect of age on nighttime sleep, there is an interaction between the effect of the variable and the effect of the method of assessment on the results. This is particularly concerning for the future assessment of the effects of other genotypes on sleep, since, arguably, the chief use of Drosophila in this field of research is to identify and study novel genetic regulators of sleep. This limitation of DAMS is unlikely to affect conclusions drawn regarding genotypes in which sleep is severely reduced both day and night because we have confirmed using video that flies are predominantly asleep during the nighttime. Indeed, researchers using DAMS in large-scale screens have successfully isolated novel mutants that have less total sleep.[9,25] However, our data suggest that conclusions drawn from studies based on DAMS data that show less drastic effects on sleep, show alteration in sleep amounts only during the daytime, change sleep architecture, or change any of these parameters in old flies should be regarded as preliminary until confirmed using video analysis. Indeed, in contrast to Drosophila *shaker* mutations, which have drastic effects on fly sleep, human disorders of altered sleep regulation are more subtle. For example, narcolepsy, in which patients are severely affected by the inability to sustain wakefulness during the day and by the inability to sustain long sleep bouts during the night, is a disorder of sleep-architecture dysregulation rather than a disorder with altered total sleep.[26] An accurate method for analyzing sleep architecture would be needed to identify and characterize a Drosophila mutant with such a phenotype.

Another variable that affects sleep architecture more than total sleep is aging.[23] The use of video analysis will allow a more accurate measure of bout fragmentation than does DAMS[23] and, possibly, will find additional effects of aging upon sleep architecture. One aspect of future research would also be to examine the arousal threshold of older animals as a function of time spent quiescent, since the 5-minute definition of sleep may not hold for aged animals.

The need to use video on a routine basis for sleep assessment introduces some practical limitations. The lighting conditions for monitoring fly behavior must be optimal in every experiment, and, currently, additional computation time is needed following data acquisition. Furthermore, at present, fewer flies can be monitored simultaneously in one incubator using video than can be monitored using DAMS. Future optimization of the hardware configuration will likely mitigate this limitation, for example, by using less expensive cameras and real-time analysis of video images. For some assessments, for example, sleep bout durations, this limitation is counteracted by the fact that video is not only more accurate for the determination of this parameter, but also shows lower variance (Table 2), thereby permitting comparisons of genotypes using fewer flies. This improved precision of video for identifying the beginning and end of a sleep bout explains our finding that the video method is more sensitive than DAMS in the detection of a homeostatic response, as assessed by sleep-latency measurements, following sleep deprivation.

Video Analysis Provides Additional Information

We have made use of the ability of video to determine the magnitude of fly movement and to identify the location of the fly in the tube. We have shown that flies utilize the space in the tube differently, depending on whether they are awake or asleep and whether it is day or night (Supplemental Figure 2). In addition, genotype can influence the preferred location in the tube, thereby likely contributing to differences in the DAMS-based sleep-measurement errors among genotypes (Table 3). There is precedence for believing that genotype could influence how the flies would distribute in the tube. Wild-type strains of flies behave differently with respect to their locomotion and use of space in the presence of food.[27] In one particular study, this difference in spatial distribution is largely explained by differ-

ences in expression levels of a single gene called *foraging*,[28] although other genetic loci modify this trait.[29]

Second, we made use of video to detect brief movements that occur during sleep bouts. These minor movements may be an indication of brief arousals in Drosophila. However, since the arousal threshold of animals during these movements has not yet been directly measured, we cannot state with certainty that these brief movements are indeed brief arousals. Based on human studies,[24] brief arousals are important in the pathophysiology of sleepiness and cognitive impairment in sleep disorders. Further study of these brief movements is therefore warranted.

There are other uses of the ability of video analysis that we have not yet fully explored. For example, the speed in which the fly is capable of moving can be measured using video. This would allow for the study of sleepy mutants that, when prodded, can nevertheless move normally. Previous studies of Drosophila sleep mutants have concentrated on short-sleeping flies because of the concern that apparent sleepy flies, which infrequently cross the path of the infrared beam, may be defective in their ability to move.

Another future direction for video analysis will be the study of sleep in different chambers. Since the current chamber used to monitor single-fly activity is small, and constrains the fly to movements that are essentially linear, the effect of particular genotypes on fly sleep may be masked or accentuated in such a chamber. That is, there may be an interaction between the genotype and the chamber. Video could be used to study sleep in multiple flies simultaneously to carefully assess the effect of social interaction on fly sleep behavior, an interaction that has been reported to have profound effects on sleep.[22] Use of video-based observations of pairs of flies have led to recent novel insights regarding mate-searching behavior in flies.[30] Finally, video could be used to detect feeding episodes[31] and, therefore, the relationship between sleep and feeding. This relationship is particularly relevant to current debates in the scientific literature, since associations have been described between acute sleep deprivation and feeding patterns[32] and between chronic short sleep deprivation and obesity.[33]

CONCLUSIONS

This study shows that the current standard in determination of sleep phenotype for Drosophila, the infrared beam break system, DAMS, misses movements, thus leading to overestimation of the true amounts of sleep. Determination of daytime sleep and of nighttime and daytime mean sleep bout duration are vastly over estimated. These errors depend on the genotype of the fly and are larger in older flies compared with younger flies. We have shown that video analysis overcomes the spatial limitations of the DAMS and represents a more accurate measure of sleep phenotype for Drosophila studies. We propose that future studies assessing sleep in Drosophila would be enhanced by use of video technology.

ABBREVIATIONS

DAMS = Drosophila Activity Monitoring System
ANOVA = Analysis of Variance
CS = Canton-S

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